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RETRACTED: Sex-Based Differences in Plasma Autoantibodies to Central Nervous System Proteins in Gulf War Veterans versus Healthy and Symptomatic Controls

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Abstract: Veterans from the 1991 Gulf War (GW) have suffered from Gulf War illness (GWI) for nearly 30 years. This illness encompasses multiple body systems, including the central nervous system (CNS). Diagnosis and treatment of GWI is difficult because there has not been an objective diagnostic biomarker. Recently, we reported on a newly developed blood biomarker that discriminates GWI from GW healthy controls, and symptomatic controls with irritable bowel syndrome (IBS) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). The present study was designed to compare levels of these biomarkers between men and women with GWI, as well as sex-specific effects in comparison to healthy GW veterans and symptomatic controls (IBS, ME/CFS). The results showed that men and women with GWI differ in 2 of 10 plasma autoantibodies, with men showing significantly elevated levels. Men and women with GWI showed significantly different levels of autoantibodies in 8 of 10 biomarkers to neuronal and glial proteins in plasma relative to controls. In summary, the present study addressed the utility of the use of plasma autoantibodies for CNS proteins to distinguish among both men and women veterans with GWI and other healthy and symptomatic control groups.

Keywords: CNS autoantibody; Gulf War illness; gender; biomarkers; proteins; myalgic encephalomyelitis/chronic fatigue syndrome; irritable bowel syndrome

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1. Introduction

Gulf War (GW) veterans have suffered from a condition known as Gulf War illness (GWI), which encompasses multiple bodily systems, including the central nervous system (CNS) [1–3]. This chronic condition affects approximately 250,000 GW veterans including a large proportion of the 40,000 women who deployed to the war. GWI has overlapping, but different, symptoms from other disorders that also affect women, including Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS), and Irritable Bowel

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Syndrome (IBS). Lack of objective diagnostic markers have minimized the ability to cohesively diagnose and treat the multitude of symptoms of GWI. Recently, we assessed a candidate plasma biomarker for GWI, and we have shown differences in individuals who meet criteria for GWI versus GW healthy, and non-veteran controls with IBS and CFS. Given the differing symptoms of GWI experienced between men and women, it was our next aim to assess possible differences in these plasma biomarkers between sexes [4–8]. Determining whether CNS autoantibodies differ between men and women with GWI and their respective controls will aid in further refining the diagnostic utility of these as plasma biomarkers.

Women who served in the 1991 GW have reported numerous health problems, many of which are gender-specific. Some studies have suggested increased rates of woman's health problems and bladder infections relative to GW-era veteran controls [9]. In the Ft. Devens Cohort (FDC) of GW veterans, GW women relative to non-veteran controls have been found to have a higher risk for diabetes but a lower risk of hypertension when compared to the National Health and Nutrition Examination Survey (NHANES) age and sex-matched cohort [8]. In contrast, GW-deployed men reported increased rates of seven chronic health conditions relative to NHANES men including significantly higher odds of reporting hypertension, high cholesterol, heart attack, diabetes, stroke, arthritis, and chronic bronchitis [8]. Because this study showed that GW men were at increased risk for medical conditions that are known risk factors for later cardiovascular and cerebrovascular disease (i.e., hypertension, high cholesterol, diabetes), GW men may show increased rates of CNS biomarkers relative to their women veteran counterparts.

However, in a recent study utilizing the large GW-era biorepository cohort (GWEBC), GW-deployed women were significantly more likely to report symptoms related to cognitive, neurological, and mood problems than GW-era women [4]. Therefore, GW women may also show increased CNS-related biomarkers compared with other women cohorts. In addition, when comparing deployed GW veteran men versus women, GW women veterans have been found to have more outpatient and inpatient health care use 5 years after deployment [10]. GW women have also been found to have higher rates of severe and moderate cases of GWI relative to their male counterparts [5,11–13].

Several factors may account for the observed sex differences in GWI and individual symptoms including sex-differences in susceptibility to neurotoxicant exposures in theatre and/or higher exposures among male personnel. As early as 1996, we speculated that chemical exposures at the time of the GW was a precipitating factor in GWI [14,15]. We demonstrated in experimental studies that combined exposure to pesticides and pyridostigmine bromide (PB) caused neurotoxicant effects that interfere with neuronal and glial cell function, leading to long-lasting and potentially neurodegenerative effects [14–16]. Neurodegeneration involves neuronal and glial cell death and the release of their contents into circulation through the breached blood brain barrier (BBB), which can then be detected through CNS autoantibodies in plasma [17]. We have previously shown BBB changes in our animal models of GWI exposures [18]. We have also previously shown that these plasma markers can be detected in low levels in individuals without significant illness but increase in those with more symptomatology [16,19].

The CNS has two types of cells: neurons and supporting glial cells. Neuronal cells contain several cytoskeletal proteins, including tau and neurofilaments [20,21]. Along with microtubules made from tubulin, they form the backbone of the axonal cytoskeleton. These proteins are involved in axonal transport of essential nutrients, amino acids, and organelles down the axon [22]. The Microtubule-Associated Proteins, (MAP)-2 and tau, promote polymerization and stabilization of microtubules in axons, cross-bridge neurofilaments, and microtubules connecting themselves to each other; this helps to maintain the cytoskeleton for axonal transport [23]. Tau proteins are localized primarily in the axon in neuronal cells. The supporting glial cells, oligodendrocytes in CNS and Schwann cells in the peripheral nervous system (PNS), produce myelin basic protein (MBP) and myelin-associated glycoprotein (MAG) that are membrane proteolipids [24]. Glial astrocytic proteins pro-

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duce glial fibrillary acidic protein (GFAP), a component of astroglia cytoskeleton, which is highly specific to the CNS and is released from the astrocyte into the extracellular space after CNS injury [25]. Calcium-binding S100B protein is also released by astrocytic glial cells (Figure 1). Many of these neuronal cytoskeletal and especially glial proteins are only found in the CNS and their autoantibodies are found in the peripheral blood, if these proteins have at some point leaked through the BBB and the immune system mounts an autoantibody response to them [18,26]. The temporal course of this BBB breach could have occurred sometime in the past in these individuals, resulting in leakage of these proteins from neurons and/or glia. So, although S100B, a marker of current BBB compromise, did not differ in our prior study in veterans with GWI, this does not mean that a BBB breach did not occur sometime in the past causing lasting detectable autoantibodies to these CNS proteins in the blood.

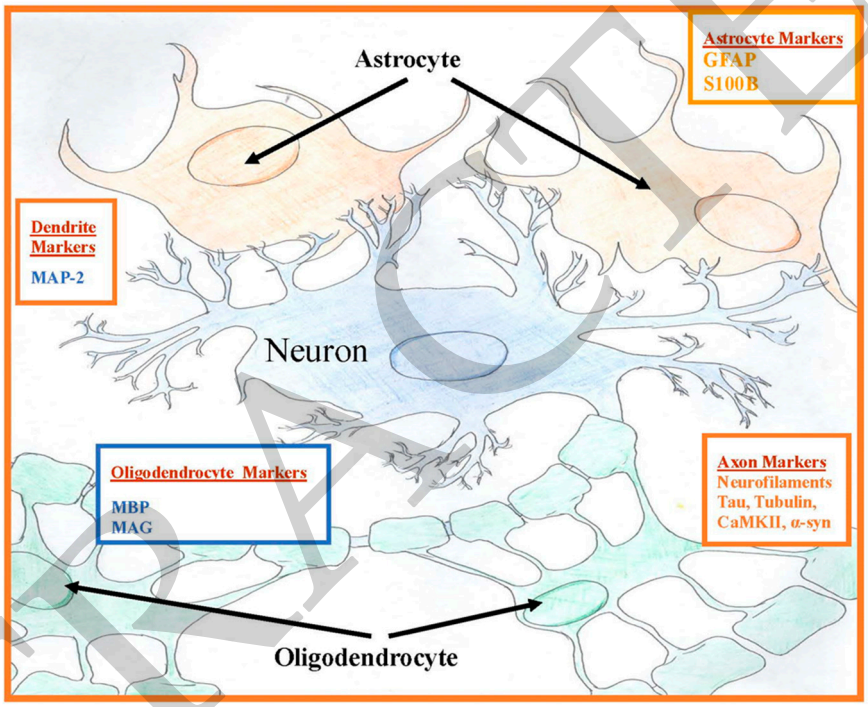


Figure 1. Central nervous system protein locations of plasma autoantibodies. Oligodendrocytes myelinate inside the central nervous system (CNS) and Schwann cells myelinate outside of the CNS.

The present study investigates the effects of gender on the presence of immunoglobulin G (IgG) class circulating autoantibodies against CNS-specific neuronal and glial proteins in the plasma of GW veterans with and without GWI as well as in symptomatic non-veteran controls (IBS, and ME/CFS). Specifically, we hypothesized that men and women veterans with GWI would differ from each other on the distribution of CNS autoantibody proteins and that GW men and women would have higher levels of CNS autoantibody proteins than other symptomatic groups with chronic illnesses including IBS and ME/CFS. Due to exposure during the war, all GW veterans could have had some level of BBB compromise as evidenced by CNS autoantibodies in plasma, but we hypothesized that those with GWI would have higher levels of these blood markers.

2. Materials and Methods

2.1. Study Population

Methods are the same as our recently published study [19]. Briefly, plasma from GW veterans were provided by the Gulf War Illness Research Consortium (GWIC), the Dynamic Modeling of GWI study from Nova Southeastern University, the Congressionally Directed Medical Research Program (CDMRP) funded study to the South Florida Veterans Affairs

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Foundation for Research and Education, Inc., the Acupuncture treatment trial, and the irritable bowel study biorepository at Harvard/BIDMC. Institutional Review Board (IRB) approval was obtained from Boston University, Nova Southeastern University and the Miami VAMC, and Harvard University.

The same standard operating procedures were used for each study site for phlebotomy, plasma separation, and aliquoting. Plasma samples were obtained from fasting subjects and stored at −80 °C. They remained frozen until analyses. Participants were consented for their respective studies using the International Conference of Harmonisation Good Clinical Practice (ICH GCP) guidelines.

The Kansas GWI criteria were used to determine cases of GWI and controls [27]. The Kansas GWI criteria require that GW veterans endorse symptoms in at least 3 of 6 symptom domains (fatigue, pain, cognitive/neurological/mood, gastrointestinal, respiratory, and skin). Veteran controls included deployed veterans from the 1991 GW who did not meet the Kansas GWI or exclusionary criteria. Kansas GWI exclusion criteria excludes individuals who report being diagnosed with another medical condition that could explain their chronic medical symptoms, including veterans with a history of prior central nervous system or major psychiatric disorders that may affect cognitive function (e.g., epilepsy, stroke, brain tumor, multiple sclerosis, Parkinson's Disease, Alzheimer's disease, schizophrenia).

Plasma samples from symptomatic controls came from prior studies of individuals with ME/CFS and IBS [28,29]. ME/CFS cases were determined by using 1994 Centers for Disease Control and Prevention (CDC) criteria [28]. IBS participants met Rome III criteria [29]. The full cohorts have been described in previous papers (GWIC, CFS, IBS, GWIC subsample [16,30–33]).

2.2. Materials

The sources of protein were also described previously [19]: Tubulin (human recombinant, Prospec Cat. # PRO-982), Microtubule-Associated Protein 2 (MAP-2, human recombinant, Origene, Cat. #TP316775, human recombinant), Tau-381 (human recombinant, Millipore Cat. #AG952), Neurofilament Protein (NFP, Prospec, Cat #PRO-523), Calmodulin Kinase II (human recombinant, CaMKII, Novus Biologicals, Cat #H000000H15-P01), Alpha-synuclein (human recombinant, AnaSpec Cat. #AS-55555), Myelin Basic Protein (MBP, human, Fitzgerald Cat. #30R-AM030), Myelin-Associated Glycoprotein (MAG, human recombinant, Sinobiological Cat. 131-86-H02H), Glial Fibrillary Acidic Protein (GFAP, human, CalBiochem Cat. #345996), S100B Protein (human, Millipore Cat. #30R-AS002). Goat anti-human IgG conjugated to horseradish peroxidase and the improved chemiluminescence reagent was obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Sodium Dodecyl Sulfate (SDS) gels, 2–20% gradient (8 × 8), and 15 mM tris-glycine were obtained from Invitrogen (Carlsbad, CA, USA). All other materials were purchased from Amersham.

2.3. Ethics Statement

Approval for the use of stored blood samples from all study sites for this study was obtained from the Duke University Health System Institutional Review Board for Clinical Investigations on 10/9/2017 and from the Boston University Medical Campus Institutional Review Board on 1/19/2018. The specific protocol components for Duke University were: Protocol ID: Pro00003202, Reference ID: 335940, Principal Investigator: Mohamed Abou Donia, Protocol Title: 'Nervous System Injury'. The specific protocol components for Boston University were Protocol ID: H-34334, Reference ID: 1288716, Principal Investigator: Kimberly Sullivan, Protocol Title: 'Novel Autoantibody Serum and Cerebrospinal Fluid Biomarkers in Veterans with Gulf War Illness.' Plasma samples were shared from IRB-approved repositories from studies at Boston University (IRB # H-32768), NOVA Southeastern University/Miami VA Medical Center (IRB # 4987.76 and IRB # 4987.75), Beth Israel Hospital/Harvard University (IRB # 2011P-000124), and the New England School of Acupuncture (IRB # 09-204).

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2.4. Procedures

2.4.1. Plasma Procedures

All sites followed the same protocol: for venipuncture, blood handling, plasma separation, aliquoting, and storage at −80 °C. The same phlebotomy and sample protocol were distributed in writing to all sites and included fasting before blood draw in the morning. All samples analyzed were baseline blood samples collected pre-intervention therapy. Samples used in this study were not previously thawed and were free of hemolysis by visual inspection.

2.4.2. Western Blot Assay

In this study, Western blot analysis was used to determine autoantibodies against specific proteins in the plasma sample of GWI cases and healthy and symptomatic controls by gender. This assay allowed the determination of the autoantibodies and associated isoforms of the antigen. As previously described, each plasma sample was analyzed in triplicate [19]. All proteins were loaded at 10 ng/lane except for IgG, which was loaded as 100 ng/lane. The proteins were denatured and electrophoresed on SDS-PAGE (gradient 4% to 20%) purchased from Invitrogen (Carlsbad, CA, USA). One gel was used for each serum sample. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham). Nonspecific binding sites were blocked with Tris-buffered Saline-Tween (TBST) (40 mM Tris (pH 7.6), 300 mM NaCl, and 0.1% Tween 20) containing 5% fat-free milk powder for 1 h at 22 °C. Membranes were incubated with serum samples at 1:100 dilutions in TBST with 3% nonfat milk powder overnight at 4 °C. After five washes in TBST, the membranes were incubated with a 1:2000 dilution of goat anti-human IgG conjugated to horseradish peroxidase (Amersham). Membranes were developed by enhanced chemiluminescence using the manufacturer's protocol (Amersham) and a Typhoon 8600 variable model recorder (GE Lifesciences, Marlborough, MA, USA). The signal intensity was quantified using Bio-Rad image analysis software version 4.5 (Hercules, CA, USA). All tests were performed with the researchers blinded to case-control and gender status of the samples.

2.4.3. Specificity of Plasma Autoantibodies

Previously, we have checked the specificity of the plasma and serum autoantibody by performing a peptide/antigen competition assay, in which the serum and plasma were spiked with the target protein or peptide [34]. The serum from random healthy controls was mixed with or without tau, MAP2, or MBP. The protein mix was centrifuged at 15,000 rpm to deplete any immune complexes. The supernatants were then carefully removed and used in Western blotting. Specificities of autoantibodies against all tested proteins were confirmed in a follow-up study [35].

2.5. Statistics

The pooled data are presented as mean ± SD for continuous variables and the number and percent of participants in each category for categorical variables. Subjects' demographic values were compared to the control groups using Students t-test continuous and chi-square for categorical variables. Mean values of autoantibodies of the GWI men and women were compared using analysis of covariance (ANCOVA), adjusting for age and race. Race was used dichotomously (Caucasian or not). Next, mean values of the GWI women were compared with GW healthy women and then repeated for GWI men and GW healthy men. Lastly, mean values of the GWI women were compared with the combined 3 control groups (GW controls, IBS, ME/CFS) using ANCOVA, adjusting for age and race; this analysis was then repeated for men. A two-sided p value < 0.05 was accepted as statistically significant for all analyses and analyses were not adjusted for multiple comparisons. Analyses were carried out using SAS version 9.4 (SAS Institute Inc. 2013. Cary, NC, USA).

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2.6. Calculations

Optical density measurement for cases and controls was divided by the concentration of serum IgG; this value for each subject was normalized to controls and expressed as change from healthy controls. Therefore, the results are expressed as mean triplicate assay values of arbitrary optical density units normalized to IgG optical density as compared to healthy controls.

2.7. CNS Autoantibody Index (CAI)

This index determines the overall neurodegenerative condition of an individual based on the level of autoantibodies in the plasma. It is calculated by adding all of the values of autoantibodies for each neural protein, and then dividing the sum by the number of the autoantibodies used. Finally, the value is multiplied by 10 to produce an easy CAI score as previously reported under the former name neurodegeneration index [19].

3. Results

3.1. Participant Demographics

The study sample included a total of 171 veterans with GWI (137 men, 34 women), a total of 56 GW male healthy controls and 4 GW women veteran healthy controls, a total of 3 male and 32 women IBS controls, and a total of 5 male and 45 women CFS symptomatic controls (Table 1). Some of the groups (Men GWI versus Women GWI, and GWI versus all combined controls) were significantly different in age and race (Caucasian or not), so all further analyses controlled for these demographic variables.

Table 1. Demographics of study sample.

	Men		Women	
Demographics	**GWI Cases (*N* = 137)**	**All Controls (*N* = 64)**	**GWI Cases (*N* = 34)**	**All Controls (*N* = 81)**
Age-Mean + SD	49.18 + 7.36	49.69 + 8.36	46.91 + 9.27	44.33 + 12.52
Race-N (%)				
Caucasian	110 (79.7)	46 (71.9)	29 (85.3)	71 (87.7)
African American	19 (13.8)	13 (20.3)	3 (8.8)	8 (9.9)
Other/Multiracial	9 (6.5)	2 (3.1)	1 (2.9)	2 (2.5)

This study reports the results of the use of our newly developed plasma neurodegenerative biomarkers to differentiate men versus women service personnel who served in the 1990/1991 GW and developed symptoms of GWI or remained healthy. In addition, analyses were conducted to detect differences between women with GWI versus the GW women and then the combined women control group (healthy GW veterans, IBS, ME/CFS) and between men with GWI vs. GW healthy men and then with the combined control men group. The levels of 10 circulating autoantibodies against neural proteins were analyzed in the plasma of men and women veterans with GWI, and with healthy GW men and women and with symptomatic men and women with CFS and IBS, which were used as controls.

3.2. Effects of Gender on the Levels of Autoantibodies against Plasma Neural Proteins

3.2.1. Autoantibody Results Analyzed by Sex for GWI Cases and GW Healthy Only

This study comprised 171 plasma samples from veterans with GWI, including 137 (80.2%) men (mean age of 49.2-years) and 34 women (19.9%) (with mean age of 46.9 years) (Table 1). Differences between men and women with respect to autoantibodies in plasma within the GWI groups were found such that males with GWI showed significantly higher levels of tubulin and MAG compared with women veterans with GWI (Table 2, Figure 2). There were no antibodies where women had higher means than the men. We also compared

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56 GW healthy males (mean age of 50.5 years) with 4 GW healthy females (mean age of 56.5 years). Although we lacked statistical power, no significant differences were found between the male and female GW healthy controls on any of the autoantibodies (data not shown).

Table 2. Autoantibody ANCOVA analysis comparing men and women with Gulf War illness (GWI).

Neural Proteins	Men (N = 137) Mean + SD	Women (N = 34) Mean + SD	p-Value
A. Neuronal Proteins			
Neurofilament triplet proteins (NFP)	3.70 + 2.75	2.40 + 1.69	0.0120
Tubulin	4.51 + 3.52	2.79 + 1.46	0.0071
Microtubule-associated tau proteins (tau)	2.99 + 1.86	2.50 + 1.53	0.1647
Microtubule-associated protein-2 (MAP)	10.11 + 4.96	8.19 + 5.18	0.0629
Calmodulin-dependent protein kinase II (CaMkII)	2.09 + 1.08	1.85 + 1.27	0.2879
Alpha-synuclein (α-syn)	2.58 + 1.62	2.28 + 1.68	0.3906
B. Glial Proteins Oligodendrocytes			
Myelin-associated glycoprotein (MAG)	5.26 + 3.44	3.57 + 2.70	0.0097
Myelin basic protein (MBP)	4.34 + 2.24	3.82 + 2.82	0.2900
Astrocytes			
Glial fibrillary acidic protein (GFAP)	4.30 + 2.37	4.01 + 2.67	0.6437
Glial S100B protein (S100B)	1.21 + 0.47	1.04 + 0.58	0.1015
CNS autoantibody Index (CAI)	41.1 + 15.7	32.4 + 16.9	0.0076

ANCOVA analyses were adjusted for age and race. Values reflect fold change relative to control.

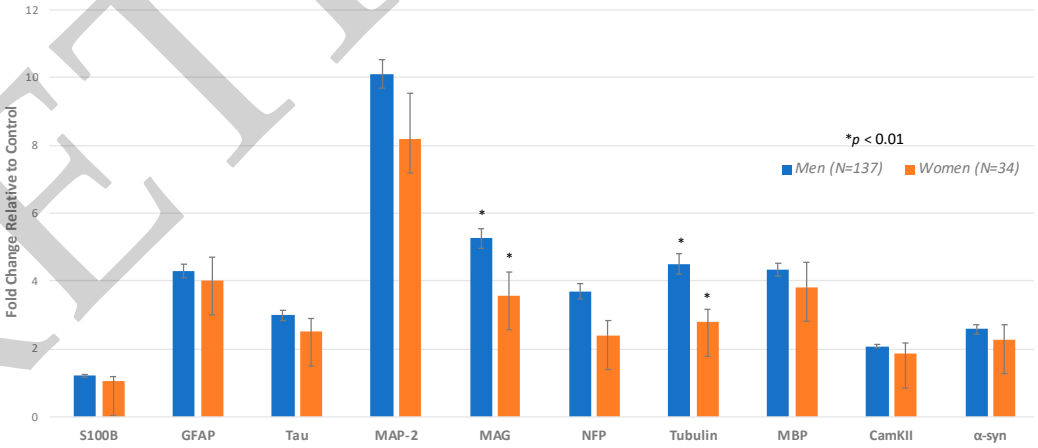


Figure 2. Analysis by sex for GWI cases only. Comparing men and women using ANCOVA adjusting for age and race. The height of the bar denotes the mean of increase in individual autoantibody biomarker +/− standard error.

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3.2.2. Autoantibody Results in Plasma of Veteran Men with GWI versus Healthy Veteran Men and then Women with GWI versus Healthy Veteran Women

The second analyses compared male veterans with GWI (*N* = 137) with GW healthy men (*N* = 56) and GWI women (*N* = 34) with GW healthy women (*N* = 4). When compared to the healthy men, GWI men had significantly higher autoantibody values for 9 out the 10 autoantibodies with the exception of S100B. When compared with healthy women, GWI women had significantly higher autoantibody values for 2 of the 10 autoantibodies, including GFAP and Tubulin (Table 3).

Table 3. Autoantibody ANCOVA analysis of GWI men and women compared to healthy Gulf War (GW) control men and women.

	Male			Female		
Neural Proteins	**GWI Cases (*N* = 137) Mean + SD**	**Healthy GW Controls (*N* = 56) Mean + SD**	***p*-Value**	**GWI Cases (*N* = 34) Mean + SD**	**Healthy GW Controls (*N* = 4) Mean + SD**	***p*-Value**
A. Neuronal Proteins						
Neurofilament triplet proteins (NFP)	3.70 + 2.75	1.91 + 2.08	<0.0001	2.40 + 1.69	1.51 + 1.05	0.0492
Tubulin	4.51 + 3.52	2.43 + 2.40	<0.0001	2.79 + 1.46	1.36 + 0.86	0.0081
Microtubule-associated tau proteins (tau)	2.99 + 1.86	1.57 + 1.32	<0.0001	2.50 + 1.53	1.61 + 1.43	0.1561
Microtubule-associated protein-2 (MAP)	10.11 + 4.96	5.03 + 5.80	<0.0001	8.19 + 5.18	5.19 + 8.22	0.1022
Calmodulin-dependent protein kinase II (CaMkII)	2.09 + 1.08	1.21 + 1.02	<0.0001	1.85 + 1.27	1.12 + 0.42	0.0555
Alpha-synuclein (SNCA)	2.58 + 1.62	1.47 + 1.33	<0.0001	2.28 + 1.68	1.37 + 1.48	0.1391
B. Glial Proteins Oligodendrocytes						
Myelin basic protein (MBP)	4.34 + 2.24	2.20 + 2.50	<0.0001	3.82 + 2.82	1.80 + 1.74	0.0724
Myelin-associated glycoprotein (MAG)	5.26 + 3.44	2.18 + 2.02	<0.0001	3.57 + 2.70	1.33 + 1.01	0.0531
Astrocytes						
Glial fibrillary acidic protein (GFAP)	4.30 + 2.37	2.38 + 2.39	<0.0001	4.01 + 2.67	1.84 + 1.12	0.0377
Glial S100B protein (S100B)	1.21 + 0.47	1.16 + 0.43	0.4174	1.04 + 0.58	1.19 + 0.36	0.6037
CNS Autoantibody Index (CAI)	41.1 + 15.7	21.5 + 17.7	<0.0001	32.4 + 16.9	18.3 + 17.6	0.0212

ANCOVA analyses were adjusted for age and race. Values reflect fold change relative to control.

3.2.3. Autoantibody Results in Plasma of Men GWI Veterans Versus All Men Controls and Women GWI Veterans versus All Women Controls

The third analysis compared male veterans with GWI (*n* = 137) and combined IBS, ME/CFS, and healthy control GW veteran males (*n* = 64), and female veterans with GWI (*n* = 34) and combined IBS, ME/CFS, and health control GW veteran females (*n* = 81) (Table 4). When compared to all male controls, men with GWI showed significantly higher

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($p < 0.01$) mean levels of 9 out of 10 autoantibodies against neural proteins (tubulin, tau, MAP-2, MBP, NFP, MAG, CamKII, α-syn, and GFAP). There was no significant difference observed for S100B. Women veterans with GWI exhibited significantly increased ($p < 0.01$) mean levels of 8 out of 10 autoantibodies when compared with the combined female control group (IBS, ME/CFS, healthy control GW veterans) including tubulin, tau, MAP-2, MBP, MAG, NFP, CamKII, and α-syn (Table 4).

Table 4. ANCOVA analysis of autoantibody differences between men with GWI and all controls and women with GWI and all controls.

	Men			Women		
Autoantibody Measure	**GWI Cases (*N* = 137) Mean + SD**	**All Controls (*N* = 64) Mean + SD**	***p*-Value**	**GWI Cases (*N* = 34) Mean + SD**	**All Controls (*N* = 81) Mean + SD**	***p*-Value**
A. Neuronal Proteins						
Neurofilament triplet proteins (NFP)	3.70 + 2.75	1.80 + 1.96	<0.0001	2.40 + 1.69	1.08 + 0.38	<0.0001
Tubulin	4.51 + 3.52	2.54 + 2.40	0.0001	2.79 + 1.46	1.90 + 1.33	0.0041
Tau proteins (Tau)	2.99 + 1.86	1.50 + 1.25	<0.0001	2.50 + 1.53	1.15 + 0.73	<0.0001
Microtubule-associated protein-2 (MAP)	10.11 + 4.96	5.01 + 5.54	<0.0001	8.19 + 5.18	4.53 + 3.78	0.0001
Calmodulin-dependent protein kinase II (CaMkII)	2.09 + 1.08	1.19 + 0.98	<0.0001	1.85 + 1.27	0.97 + 0.43	<0.0001
Alpha-synuclein (α-syn)	2.58 + 1.62	1.43 + 1.25	<0.0001	2.28 + 1.68	0.98 + 0.44	<0.0001
B. Glial Proteins Oligodendrocytes						
Myelin basic protein (MBP)	4.34 + 2.24	2.15 + 2.37	<0.0001	3.82 + 2.82	1.36 + 0.55	<0.0001
Myelin-associated glycoprotein (MAG)	5.26 + 3.44	2.19 + 1.90	< 0.0001	3.57 + 2.70	2.19 + 1.31	0.0004
Astrocytes						
Glial fibrillary acidic protein (GFAP)	4.30 + 2.37	2.51 + 2.40	<0.0001	4.01 + 2.67	3.11 + 2.41	0.1379
Glial S100B protein (S100B)	1.21 + 0.47	1.19 + 0.45	0.6833	1.04 + 0.58	1.15 + 0.39	0.0952
CNS Autoantibody Neurodegeneration Index (CA_NDI)	41.1 + 15.7	21.5 + 16.8	<0.0001	32.4 + 16.9	18.4 + 7.24	<0.0001

ANCOVA analyses were adjusted for age and race. Values reflect fold change relative to control.

3.2.4. CAI Values by Gender in Men and Women GWI Cases versus Men and Women Controls

When all autoantibodies were combined into the CAI score, men with GWI had the highest values of total combined autoantibodies, as indicated by a CAI score of 41.1, and women with GWI showed a CAI score of 32.4. These values were statistically significantly different by ANCOVA, adjusting for age and race ($p = 0.007$). The CAI score of the GW control men had a mean of 21.4, which was statistically lower than the GWI men ($p < 0.0001$) (Figure 3). The CAI score of the GW control women had a mean score of 12.0, which was statistically lower than the GWI women ($p < 0.021$). The combined male control group had a mean CAI of 21.5, which was statistically significantly lower than the GWI male

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group ($p < 0.0001$). In addition, the combined women control group had a mean CAI of 18.7, which was also significantly lower than the GWI women group ($p < 0.0001$). In this study, our results of both men and women with GWI showed that their CAI values were higher than 90% of controls.

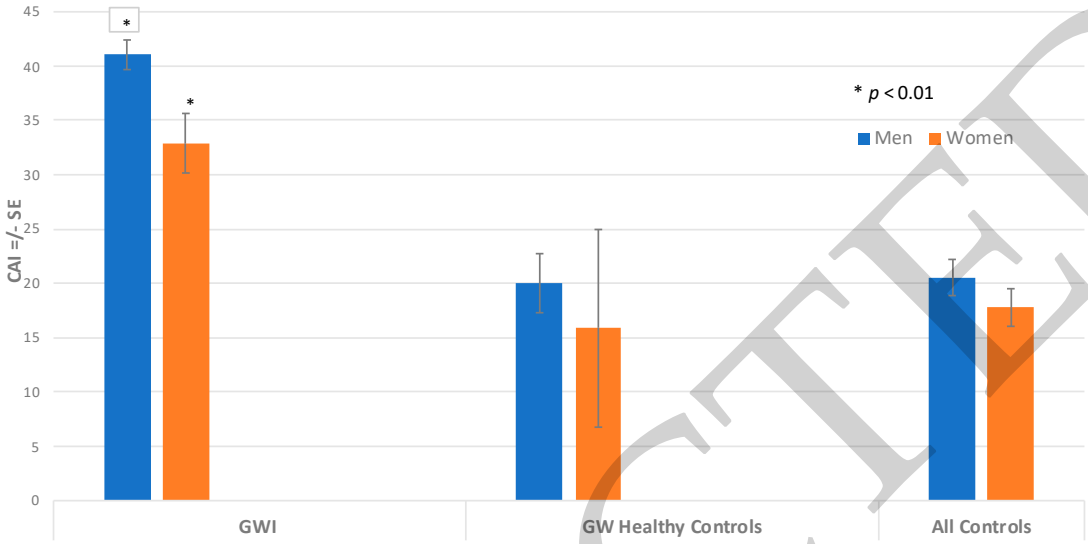


Figure 3. CNS Autoantibody Index (CAI) for Men and Women. Comparing CAI for men and women using ANCOVA, adjusting for age and race. The height of the bar denotes adjusted mean CAI +/− standard error. CAI for GWI is significantly different than CAI for GWI women (* $p < 0.01$).

4. Discussion

This study, using our previously validated plasma CNS autoantibody biomarkers, shows that there was a difference between men and women veterans with GWI, with male veterans having significantly higher levels for 2 out of the 10 autoantibodies (tubulin, MAG) that are related to axonal and oligodendrocyte functions (Figure 2). When combining all of the autoantibodies into the CNS autoantibody neurodegenerative index (CAI), a significant difference between men with GWI and women with GWI was observed, such that men with GWI had more overall autoantibody protein burden and a significantly higher CAI value. Our second set of analyses compared male veterans with GWI to male GW healthy controls and then repeated the analyses for women veterans with GWI compared with healthy GW women. Our next analyses compared male veterans with GWI to all male controls from our prior study (healthy GW veterans, non-veterans with IBS or ME/CFS) [19]. We then performed the same analyses comparing women veterans with GWI to the combined all women control group (non-veterans with IBS and ME/CFS). The results showed that men with GWI had significantly higher levels of autoantibodies for 9 out of the 10 autoantibodies when compared with male healthy GW veterans or with the combined male control group. Women with GWI showed significantly higher values for 2 out of the 10 autoantibodies when compared with women healthy GW veterans and with 8 out of 10 autoantibodies when compared with their respective combined women control group (nonveterans with IBS and ME/CFS). The CAI analyses comparing GWI men with their male control group and GWI women with their respective control group also showed significantly higher values for the GWI men and women, with double the values compared with respective controls. These results add to our prior findings that suggested using a cutoff of 30 or more on the CAI score would distinguish between GWI and other chronic disorders. These results indicate that this cutoff corresponds with both men and women veterans with GWI.

Although men and women veterans with GWI had higher autoantibody levels than their respective control groups, they only differed from each other on two CNS autoantibody proteins. However, the total combined CAI score was a significantly higher value for

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the men with GWI. The two significantly different proteins included tubulin and MAG, which are related to axonal and oligodendrocyte functioning, which have been associated with history of mild traumatic brain injuries (mTBI) and to chemical weapons exposures. These results correspond with our prior studies showing increased rates of mTBI and chemical weapons exposures during the war, particularly in male veterans with GWI [30,36,37]. In fact, this multiple-hit hypothesis suggested those with both mTBI and chemical weapons exposure would report more chronic health symptoms that may be related to these axonal and myelin-related pathobiological markers. Correspondingly, Carney et al. compared combat experiences, occupational, and other service-related exposures and health care use of GW male and female veterans and reported similar military experiences but with male veterans more often participating in combat related activities. However, no significant gender differences were found in exposure to solvents/petrochemicals, infectious diseases, neurotoxins, heat stress, trauma, or radiation [38]. In addition, the women veterans with GWI differed from the GW women healthy controls on two markers, including a glial marker (GFAP) and a neuronal cytoskeletal marker (Tubulin). These results suggest that women with GWI appear to be showing more neuronal cytoskeletal and neuroinflammatory changes when compared to healthy GW controls or women with IBS or ME/CFS. The male veterans with GWI differed from the GW healthy males with higher values on all autoantibodies except for the acute glial BBB marker S100B and the CAI value, which was twice that of the controls. When the women with GWI were compared with the combined control group, they showed higher levels of autoantibodies on all markers except the glial markers GFAP and S100B. Again, their CAI value was nearly twice that of the combined controls. Similar to the prior analysis, the male veterans with GWI did not differ from their respective controls for the glial activation and acute BBB marker S100B. This suggests that male GWI veterans may be showing more chronic glial activation, neuronal damage, and neuroinflammation than their male control healthy and symptomatic counterparts with IBS and ME/CFS because S100B is a marker of current BBB disruption and GFAP is a marker of current neuroinflammation [39,40]. This is because GFAP is secreted by activated astrocytes, which leads to neuroinflammation [41–43].

With regard to integrating our findings within the larger scope of GWI research relevant to sex differences, it has been shown that there are differences in immune inflammatory markers and in overall rates of health symptoms between GW veteran men and women [5,8,13,44–46]. The current study adds to the literature in that GW veteran men have higher overall values of autoantibodies (as assessed by the CAI ratio), and that the findings are consistent with men and women potentially experiencing increased autoantibodies to a similar degree, but with a lesser increase in autoantibodies in women associated with greater symptom burden. These results suggest that further study of the sensitivity of the CAI cutoff of 30 for both men and women veterans with GWI is warranted.

Autoantibodies to neural proteins in addition to being blood biomarkers are also indications of neurodegeneration and may explain the mechanisms of brain diseases and aging effects [26,47]. Currently, the only consistent risk factors for GWI are chemical exposures and a history of mTBI [30,36,37,48–51]. These chemicals include: pyridostigmine bromide, pesticides including the insecticides permethrin and lindane as well as the insect repellant, DEET (*N*,*N*-diethyl-*m*-toluamide), and organophosphate (OP) insecticides and nerve gases, sarin and cyclosarin, which are now known to adversely affect the CNS in significant or combined dosages [3,14,15,48,51–58].

Investigations into the mechanisms by which OP compounds cause neurodegeneration have established that OPs increase the activity and expression of calcium-calmodulin Kinase II (CaMKII) that causes hyperphosphorylation of neural proteins, leading to their aggregation and slowing of axonal transport, resulting in neuronal cell death [51,59–62]. In agreement with this is our prior finding that airline crews who were exposed to OPs developed autoimmune antibodies to neural proteins [34]. Increased autoantibodies to neuronal proteins such as Tau, NFP, MAG, MBP, and GFAP are consistent with the brain imaging study in another cohort of airline crews which showed decreased brain white

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matter microstructure and cerebral perfusion, which may be potential causes of cognitive impairments and mood deficits reported by the aircrews [63]. The significance of this finding is that GFAP is a marker of astrocyte activation and astrocytes can directly interact with and restrict brain vasculature including capillaries that are involved in cerebral perfusion and neuroinflammation.

Health symptom complaints reported by GW veterans are consistent with sequelae following exposure to pesticides such as organophosphates, pyrethroids, and DEET [51,64,65]. Increased autoantibodies against neurofilament, tau, tubulin, and myelin basic proteins, which are biomarkers for myelin and neuronal cytoskeletal disruptions including microtubule instability, axonal degeneration, and altered axonal transport, have been found in many cell and animal studies of toxicant-induced models of GWI [66–74]. Our results are consistent with previous reports showing increased various autoantibodies in a smaller and then larger study of GW veteran blood samples [16,19,75–78]. To our knowledge, this is the first study to assess the sex effects of these autoantibodies in veterans with GWI compared with healthy and symptomatic comparison groups.

In summary, the results of the present investigation on the effect of gender on blood biomarkers showed that the levels of autoantibodies were significantly higher for both men and women GW veterans compared to controls [19]. When there was a sex difference, male veterans with GWI exhibited higher levels of autoantibodies as well as the overall CAI score than women veterans with GWI. This is consistent with the situation in the war theatre, that although men and women had similar military experiences, men more often participated in combat and had higher rates of mTBI [30,36–38]. This is not to suggest that women veterans do not have increased levels of other symptoms of this multi-factorial disorder but rather that male veterans appear to be showing more CNS autoantibody differences than women veterans as indicated by higher CAI total values.

Limitations

Like most studies, our study had limitations. We had small group sizes in some analyses that reduced our power to see differences between the groups. This was especially true for the women's comparisons. Because the main focus of this study was to assess GWI vs. symptomatic control groups, we did not have a non-veteran healthy control group for comparison, which may have provided more clarity to the results had that group been included. In this study, we used the Kansas criteria for GWI that should have excluded known cases of disorders including Alzheimer's Disease (AD), Parkinson's Disease (PD), and other chronic illnesses that could have accounted for their chronic symptoms [27]. In this study, we demonstrated associations between autoantibodies, particularly in GWI veterans, as compared to healthy and symptomatic controls stratifying by sex. However, the temporal relationship remains unclear between these conditions and production of autoantibodies. More research is needed to determine if these are blood-based CNS proteins from exposures 30 years ago or if there are ongoing CNS changes that are eliciting these autoantibody proteins. Other larger studies with women veterans would also help further validate the pathobiological impact of GWI on women veterans.

A major strength of our study is that it represents both healthy and symptomatic GW veteran groups as well as symptomatic non-veteran controls with ME/CFS or IBS. This suggests that both men and women veterans with GWI differ not only from their healthy GW veteran controls but also have more CNS differences than other groups of men and women with chronic multi-symptom illnesses. In addition, the CNS autoantibody analyses were performed with the laboratory staff blinded to the case status of all participants.

5. Conclusions

This year marks the 30th anniversary of the 1990/1991 Gulf War. Throughout most of this time, there has been a lack of diagnostic tools to accurately diagnose the disorder, which has hindered both accurate diagnoses and much-needed treatments for veterans. Our laboratory was one of the first to propose and document that GWI is related to

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chemical exposures during the war and that those exposures adversely affected the CNS. In addition, we have documented differences in autoantibodies between GWI and GW-healthy and symptomatic controls. This study documents that there is a sex effect for these groups with men and women with GWI showing higher levels of autoantibodies than their respective control groups and with male veterans with GWI showing the greatest burden of autoantibodies. After further validation, we are hopeful that our newly developed CNS Autoantibody Neurodegeneration Index (CAI) summary score cutoff of 30 or more can be utilized to develop objective diagnostic markers of GWI and to compare treatment trial effectiveness for both men and women GW veterans.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Boards of Duke University Health System Institutional Review Board for Clinical Investigations on 10/9/2017 and from the Boston University Medical Campus Institutional Review Board on 1/19/2018.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data are available upon request from the corresponding authors.

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Conflicts of Interest: The authors report no relationships that could be construed as a conflict of interest. The authors alone are responsible for the content and writing of the paper. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense.

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